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Recognition of the Beta-Subunit of Human Chorionic Gonadotropin and Sub-Determinants by Target Tissue Receptors

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The beta-subunit of human chorionic gonadotropin, purified immunochemically to eliminate undissociated human chorionic gonadotropin, induced testosterone production by mouse Leydig cells at concentrations 400-fold higher than human chorionic gonadotropin. Steroidogenesis was also stimulated by a synthetic fragment of the beta-subunit of human chorionic gonadotropin conforming to the peptide sequence residues 39–71, whereas peptide sequence residues 39–56 and three *C*-terminal fragments (residues 115–145, 111–145 and 101–145) failed to cause steroidogenesis. These studies suggest the presence in the beta-subunit of human chorionic gonadotropin of determinants recognized by the tissue receptors, a part of these determinants residing between amino acid residues 57–71.

Human chorionic gonadotropin, though of human origin, acts on ovarian and testicular tissues of a wide variety of mammalian species. The hormone induces ovulation and steroidogenesis in the female and promotes the production of testosterone in the male. Leydig cells from the testis carry receptors for human chorionic gonadotropin, whose properties and characteristics are similar to those present in the ovaries (Lee & Ryan, 1973; Catt & Dufau, 1973). These cells are a good model for the study of hormone–tissue receptor interaction linked functionally to the consequences of it, namely the production of testosterone. This bioassay system developed by Dufau *et al.* (1974) and Van Damme *et al.* (1974) is highly sensitive and can measure as low as 1.5–2.25 pg of human chorionic gonadotropin. We have used this system to seek information on the question as to whether the beta-subunit of the hormone and its subfragments are recognized by the receptors and can cause steroidogenesis. This question has interest from the point of view of the determinants involved in the gonadotropin action and has also implications on the current approaches to the development of anti-(human chorionic gonadotropin) vaccines for the control of fertility (Talwar *et al.*, 1976; Stevens, 1976; Hearn, 1976).

Materials and Methods

Hormones and derivatives

Human chorionic gonadotropin with biological activity of 10800i.u./mg was supplied by Dr. Tsong of the Population Council, New York, NY, U.S.A. The highly purified beta-subunit of human chorionic gonadotropin was absorbed with anti-(alpha-human chorionic gonadotropin) immunoabsorbant to remove trace amounts of free alpha-subunit and undissociated human chorionic gonadotropin (immunochemically suppressed beta-subunit of human chorionic gonadotropin).

The reduced carboxymethyl derivative of the asialo-beta-subunit of human chorionic gonadotropin and the core fragment of asialo-beta-subunit of human chorionic gonadotropin (residues 1–40 linked to residues 50–114) retaining intact disulphide bonds after digestion with thermolysin were made available by Dr. S. Birken and Dr. R. E. Canfield, College of Physicians and Surgeons, Columbia University, New York, NY, U.S.A.

Synthetic peptides

The following five peptides of the beta-subunit of human chorionic gonadotropin synthesized according to the sequence proposed by Morgan *et al.* (1975) were prepared by Dr. Karl Folkers of Texas University at Austin, TX, U.S.A., and made available through I.D.R.C. and the International Committee for Contraception Research of the Population Council, New York. These were: (i) *C*-terminal 31-amino acid peptide (residues 115–145); (ii) *C*-terminal 35-amino acid peptide (residues 111–145); (iii) *C*-terminal 45-amino acid peptide (residues 101–145); (iv) core 18-amino acid peptide (residues 39–56); (v) core 33-amino acid peptide (residues 39–71 in which cysteine at residue 57 was replaced by aminobutyric acid).

The synthetic fragments, subunit preparations, immunochemically suppressed beta-subunit of human chorionic gonadrotropin and human chorionic gonadotropin were dissolved in chilled 50mM-phosphate buffer (pH 7.4) containing 0.15mM-NaCl and 0.1% bovine serum albumin (w/v).

Leydig-cell assay

The procedure was essentially that of Van Damme *et al.* (1974) with some modifications described elsewhere (Das *et al.*, 1978).

Results

The dose–response relationship between human chorionic gonadotropin and the square root of the amount of testosterone produced by Leydig cells is shown in Fig. 1. A linear response was observable between 0.28 and 0.96fmol of the hormone per tube (0.93–3.19pM). The beta-subunit of human chorionic gonadotropin passed through an anti-(alpha-human chorionic gonadotropin) immunoabsorbent to remove undissociated human chorionic gonadotropin was without any biological activity at comparable concentrations. However, when the subunit was used at higher concentrations (200–1000-fold), there was a significant dose-dependent stimulation of the testosterone production. The dose–response curve of the beta-subunit had a parallel slope to that of human chorionic gonadotropin. An almost equivalent response was observed with the immunochemically suppressed beta-subunit at 400-fold higher concentrations than human chorionic gonadotropin.

To check whether the observed biological activity was indeed intrinsic to the beta-subunit of human chorionic gonadotropin, two types of experiments were carried out. In one of these, the native molecule was chemically denatured and then tested for its ability to stimulate the Leydig cells. The beta-subunit of human chorionic gonadotropin has 12 cysteine residues, but no measurable free thiol groups. Reduction and alkylation results in conformational changes that abolish the biological activity of the hormone. The reduced carboxymethyl derivative of the asialo-beta-subunit of human chorionic gonadotropin was found to have no steroidogenic potency (Fig. 1).

Another approach was to use synthetic fragments conforming to the amino acid sequence of the beta-subunit of human chorionic gonadotropin. These will be devoid of contamination with trace amounts of the undissociated hormone. Five synthetic peptides of various sizes and a core fragment of the asialo-beta-subunit of human chorionic gonadotropin cleaved enzymically were used. Results in Fig. 2 indicate that the *C*-terminal 31-, 35- and 45-amino acid fragments do not possess any biological activity up to the concentrations investigated (1.0nmol/tube, i.e. 3.33μM), which are approx. 1000-fold higher than the highest amount of immunochemically suppressed beta-subunit of human chorionic gonadotropin used for the studies reported in Fig. 1. The thermolysin-cleaved core unit had low, but perceptible, biological activity. It is noteworthy that the synthetic

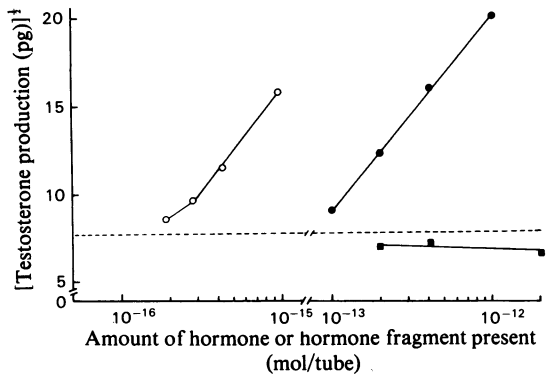

Fig. 1. *Effect of human chorionic gonadotropin* (○) *and immunochemically suppressed beta-subunit of human chorionic gonadotropin* (●) *on testosterone production by mouse Leydig cells*

The values are means for quadruplicate determinations. The broken line denotes the basal production of testosterone by the Leydig cells in the absence of hormone. The reduced carboxymethyl derivative of the asialo-beta-subunit of human chorionic gonadotropin (■) did not cause stimulation of steroidogenesis at the concentrations tested.

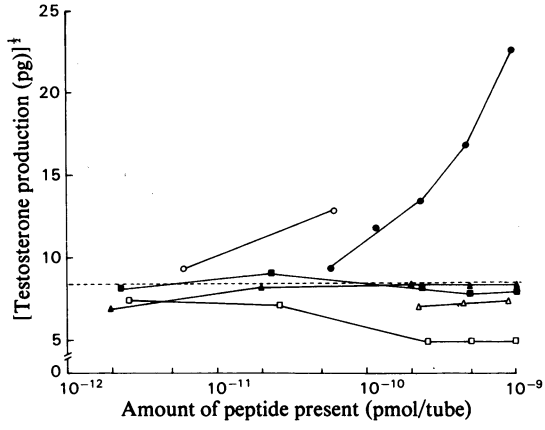

Fig. 2. *Effect of synthetic peptides and an enzyme-cleaved fragment of the beta-subunit of human chorionic gonadotropin on testosterone production by mouse Leydig cells*

Symbols: □, *C*-terminal peptide residues 115–145; ■, *C*-terminal peptide residues 111–145; ▲, *C*-terminal peptide residues 101–145; △, core 18-amino acid peptide residues 39–56; ●, core 33-amino acid peptide residues 39–71; ○, thermolysin-cleaved fragment of the asialo-beta-subunit of human chorionic gonadotropin (fragments 1–40 linked to fragments 50–114). The broken line indicates the basal level of testosterone production. The values are means for quadruplicate determinations.

fragment conforming to the sequence of residues 39–71 stimulated the Leydig cells to produce significant amounts of testosterone. A dose-dependent response with this 33-amino acid peptide was observed between 50 and 1000pmol/tube (0.166–3.33μM). On the other hand, the peptide of residues 39–56 did not possess any steroidogenic activity at comparable concentrations.

Experiments were carried out to test whether the fragment of residues 39–71, after incubation with the alpha-subunit of human chorionic gonadotropin under conditions favouring the association of alpha- and beta-subunits of human chorionic gonadotropin (Aloj & Ingham, 1977), improves its biological potency. This was, however, not found to be the case.

Discussion

Human chorionic gonadotropin, like other gonadotropins, is a glycoprotein composed of two subunits. The alpha-subunit is nearly identical in human chorionic gonadotropin, human lutropin, follitropin and thyrotropin (Pierce *et al.*, 1976); the beta-subunit imparts in each case individuality to the action of these hormones. This notion is substantiated by generation of respective hormonal activities on hybridization of the beta-subunits of different hormones to hetero-alpha-subunits (Pierce *et al.*, 1971; Reichert *et al.*, 1974; Pernollet *et al.*, 1976; Aloj & Ingham, 1977). Dissociation of human chorionic gonadotropin into individual subunits results in a large decrease in biological activity, as measured by ventral-prostate weight gain (Canfield *et al.*, 1971), radio-receptor assay (Kammerman *et al.*, 1972) and progesterone production by cultured granulosa cells (Channing & Kammerman, 1973). Several investigators have in the past ascribed various types of biological activities to the beta-subunit of gonadotropins (Farmer *et al.*, 1973; Rao & Carman, 1973; Yang *et al.*, 1972, 1973: Muralidhar & Moudgal, 1976). However, owing to the strong tendency of the subunits to reassociate, the question of whether the observed low biological activity is due to the subunit itself or to small residual amounts of the undissociated gonadotropin has been examined (Rayford *et al.*, 1972; Morgan *et al.*, 1974).

In the present study, the beta-subunit of human chorionic gonadotropin used was not only chemically fractionated, but also immunochemically purified to eliminate alpha-human chorionic gonadotropin and undissociated human chorionic gonadotropin in the preparation. Furthermore, synthetic fragments of the beta-subunit of human chorionic gonadotropin that are free of native hormone were also utilized to confirm the basic tissue.

Total human chorionic gonadotropin, in which the subunits are in an associated state, was highly potent and induced steroidogenesis at concentrations as low as 0.19fmol/tube (0.63pM). The dissociation of native molecule into the subunits resulted in considerable loss of biological activity. Nevertheless, the beta-subunit alone was not totally devoid of biological activity. It was able to cause steroidogenesis at concentrations several-hundred-fold higher than the native molecule. The observed biological activity of the beta-subunit was lost by reduction and alkylation. A possible contribution by trace contamination of human chorionic gonadotropin in the subunit preparations can be excluded on the ground that a 33-amino acid synthetic peptide of the beta-subunit of human chorionic gonadotropin was also able to stimulate testosterone production in the system. Although the beta-subunit of human chorionic gonadotropin has adequate information to stimulate steroidogenesis in the Leydig cells, the efficiency with which the hormonal message is transmitted is much better with the associated alpha- and beta-subunits. Dissociation of lutropin and human chorionic gonadotropin results in limited conformational changes as indicated by the decrease in the beta-sheet and alpha-helical content as well as exposure of 2 to 3 tyrosine residues to the solvent (Garnier *et al.*, 1974; Salesse *et al.*, 1975; Ingham *et al.*, 1976). The native gonadotropin enhances the fluorescence of 8-anilinonaphthalene-1-sulphonate, a hydrophobic fluorescence probe, whereas the isolated subunits do not do so (Aloj *et al.*, 1973). The association of beta-subunit with the alpha-subunit seems thus to lead to new conformations which enhance the efficiency of the determinants on the beta-subunit of human chorionic gonadotropin to interact with the tissue receptors and stimulate steroidogenesis.

Within the beta-subunit the determinants, or at least a part of the epitopes recognized by the receptor, which are instrumental in inducing functional response in cells, were found to reside in the sequence of residues 39–71. Since the fragment of residues 39–56 was not biologically active, it can be inferred that the sequence of residues 57–71 is important for the biological activity. The biological potency of the fragment of residues 39–71 was not improved on incubation with the alpha-subunit of human chorionic gonadotropin, suggesting that this part of the molecule may not possess the association sites with the alpha-subunit.

The *C*-terminal moiety of the beta-subunit of human chorionic gonadotropin is of special interest because the last 30 amino acids are not present in the best-subunit of lutropin and are unique to the beta-subunit of human chorionic gonadotropin. This part of the molecule, however, does not seem to be recognized by the receptors on the target cells. The peptide, up to a concentration of 537.6nM, did not compete with the binding of ^{125}I-labelled human chorionic gonadotropin to sheep corpora luteal receptors (Talwar *et al.*, 1978). Similar indications

are available from the present data on *C*-terminal 35- and 45-amino acid fragments. The determinants of the beta-subunit of human chorionic gonadotropin recognized by the receptors on target cells are likely to reside in the core part of the molecule and not at the tail end. This does not, however, preclude the possibility of antibodies against *C*-terminal fragments interfering in the biological activity of human chorionic gonadotropin by causing steric hindrance and binding at sites distant from the receptor recognition site. The frequency with which such antibodies are formed should, however, be carefully investigated.

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